XCIII. THE BIURET METHOD OF ESTIMATING ALBUMIN AND GLOBULIN IN SERUM AND URINE.

By JOSEPH FINE.

From the Laboratory of Chemical Pathology, St Bartholomew's Hospital.

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QUANTITATIVE determinations of serum and urinary proteins are of considerable clinical importance; that such estimations are made relatively infrequently is probably due to the lack of a simple yet trustworthy technique.

The methods of Esbach and Aufrecht are notoriously crude, while the gravimetric and the Kjeldahl methods are too laborious for ordinary clinical purposes. Of the colorimetric procedures the biuret method and that of Wu [1922] are both reliable, the error usually not exceeding 5 % in experienced hands. The latter however has the disadvantage that the calculations are somewhat involved, and it is moreover more lengthy than the biuret method modified as described below.

THE BIURET METHOD AS HITHERTO CARRIED OUT.

Protein is separated by salting out or by coagulation, and the precipitate is treated with NaOH and $CuSO_4$. The resulting violet colour is compared with that of a standard solution (usually of protein) similarly treated.

The method was first introduced by Riegler [1914] who applied it to urine and used as a standard pure dry serum. Autenrieth [1915] used a standard serum solution diluted with normal urine, and also extended the method successfully to the estimation of proteins in ascitic fluid and serum [1917]. Hiller [1927] proposed as standard a solution of 0·266 % pure biuret.

DISCUSSION OF THE BIURET METHOD.

1. *Choice of a standard.* In the writer's work biuret has been unsatisfactory for two reasons. Firstly, it is difficult to obtain pure biuret; a sample from a well-known firm contained about 55 % biuret, the remainder being urea and NH_4Cl. Until this was ascertained the protein results were consistently about double the correct values.

Secondly, the tint of the treated biuret solutions differs materially from that of protein solutions, leading to an increased personal error. The use of serum as standard obviates this difficulty.

When preserved with chloroform the serum-protein keeps for long periods; no change has been observed in 6 months.

As a standard the serum is diluted to contain 0·24 % protein. On keeping, a small deposit separates, but this is immaterial for the same result is obtained whether or not the deposit is resuspended.

2. *Biuret colour values of albumin and globulin.* Autenrieth [1917] states that on treatment equal quantities of albumin and globulin yield violet solutions of practically the same intensity. This was confirmed as follows. Solutions of albumin and globulin were obtained from a sample of serum by half saturating

with ammonium sulphate and filtering. The globulin deposit was dissolved in NaOH; the albumin in the filtrate was precipitated with $CCl_3.COOH$, the mixture centrifuged and the albumin redissolved in NaOH. The protein contents of the albumin and globulin solutions were determined by the Kjeldahl method.

Varying amounts of the two solutions were then taken and the total protein content in each estimated in duplicate by the biuret method (see technique below) using as standard first the albumin solution and then the globulin solution. The results are shown in Table I.

Table I.

Volume of solution estimated	Protein content (Kjeldahl)	Protein content, biuret method: Albumin standard			Protein content, biuret method: Globulin standard		
ml.	mg.	mg.	% error		mg.	% error	
Albumin 2	10·4	10·4	0		9·9	−5	
2·5	13·0	13·0	0		12·6	−3	
3	15·6	15·2	−3	Mean error	14·7	−6	Mean error
3·5	18·2	17·3	−5	−2·5 %	16·9	−7	−5·6 %
4	20·8	20·2	−3		19·4	−7	
4·5	23·4	22·5	−4		21·9	−6	
Globulin 2	7·4	8·06	+9		7·4	+1	
3	11·1	10·9	−2	Mean error	10·4	−6	Mean error
4	14·8	15·2	+3	+4 %	14·8	0	−1·4 %
5	18·5	19·1	+3		18·5	0	
6	22·2	22·75	+2·5		22·2	0	

Table I shows that out of 22 estimations by the biuret method the error is between 0 and 5 % in 16 cases and between 5 and 10 % in 6 cases, and that accuracy is greatest when an albumin standard is used to estimate albumin, and a globulin standard used to estimate globulin.

When a globulin standard is used to estimate albumin the results tend to be low, and when albumin is used to estimate globulin the results tend to be high. These results suggest that globulin has a biuret colour value slightly higher than that of albumin.

The difference, however, is small and is within the range of experimental error, particularly as there was a slight difference in tint between the colorimetric solutions obtained from the albumin and globulin standards (a difference infrequently obtained in other estimations).

3. *Discrepancies between albumin results obtained by different salting-out methods.* Howe [1923] states that all the proteins of plasma except albumin are precipitated by

(1) $1{\cdot}5M$ Na_2SO_4 (21·3 %),
or (2) $2M$ $(NH_4)_2SO_4$ (26·4 % or "half saturated"),
or (3) $2{\cdot}375M$ $MgSO_4$ ("saturated").

Race [1932] points out that in Howe's own work these three methods do not give the same albumin figures and my own results confirm this.

Table II gives the percentages of albumin in various sera after salting out with Na_2SO_4 and $(NH_4)_2SO_4$ respectively and applying the biuret method.

Out of a total of 9 estimations only once has salting out with ammonium sulphate led to a lower result than with sodium sulphate; in all other cases the results with ammonium sulphate have been higher as illustrated in Table II.

Table II. *Albumin percentages of serum.*

Serum	Using 21·3 % Na_2SO_4 %	Using 26·4 % $(NH_4)_2SO_4$ %	% difference
Ox	3·80	4·40	+16
Human H.	4·40	4·75	+ 8
,, B.	4·55	5·00	+10
,, T.	4·44	5·06	+14
,, W.	4·73	5·20	+10
Mixed human	4·80	5·70	+19

Fig. 1 illustrates the amount of protein precipitated in three sera by increasing concentrations of ammonium sulphate and shows that to obtain

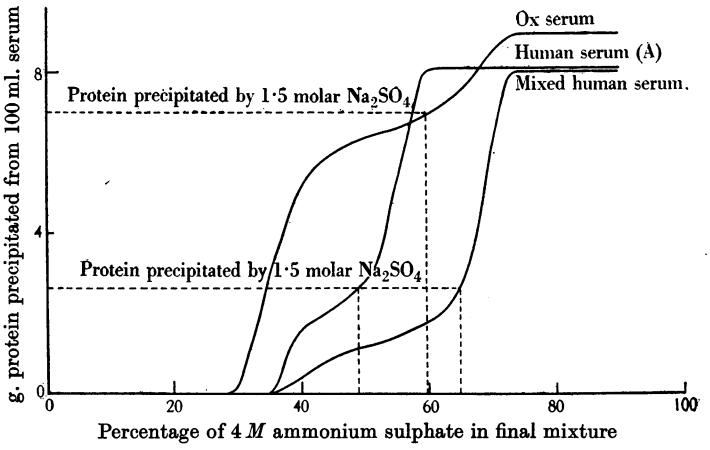

Fig. 1.

protein precipitates equal to those brought down by 21·3 % sodium sulphate the respective concentrations of 4*M* ammonium sulphate required were 48, 59 and 65 %.

It is clear that 2*M* ammonium sulphate is not invariably equivalent to 1·5*M* sodium sulphate in fractionating proteins from serum.

If globulin is defined as that portion of the serum proteins which is precipitated by 2*M* ammonium sulphate, salting out by 1·5*M* sodium sulphate may give different results for "globulin", and further such differences are irregular.

Similarly if globulin is defined as that portion of the serum proteins which is precipitated by 1·5*M* sodium sulphate, salting out by 2*M* ammonium sulphate will often give different results.

It is important that this discrepancy should be generally realised, and that some convention should be adopted in defining "globulin". In the meanwhile in the writer's work ammonium sulphate has been employed, because it avoids the need for working at 37° which is essential for salting out by sodium sulphate in Howe's technique.

4. *Time required for the method.* Any accurate method of protein estimation involves many hours, and even the colorimetric methods are greatly delayed in estimating albumin owing to the customary procedure of allowing the Na_2SO_4-protein mixture to stand for 3 hours before filtering. I have found that this filtration can be completed in 15 minutes without affecting the result if

the salt + protein is filtered immediately after mixing and refiltered as soon as the filtrate comes through clear.

Performed in this way the estimation of albumin and globulin can be completed in less than 45 minutes.

Technique of modified method for serum proteins.

1. *Total proteins.* With a blood pipette calibrated "to contain", measure 0·2 ml. serum into 2 or 3 ml. distilled water in a 15 ml. graduated centrifuge-tube: add more distilled water up to 5 ml. and 10 % $CCl_3.COOH$ up to 10 ml.: mix, centrifuge for 3 minutes at about 3000 revolutions per minute.

Decant the clear supernatant fluid: add 1 ml. 30 % NaOH and about 3 ml. distilled water and shake: when the protein has dissolved add more water up to 9 ml. then 5 % $CuSO_4$ up to 10 ml. Shake for 10 seconds.[1] Centrifuge as before. Decant the supernatant fluid for comparison in the colorimeter with the standard.

2. *Albumin.* With an Ostwald pipette calibrated to "blow out", measure 0·5 ml. serum into a test-tube and add 9·5 ml. of 27·79 % ammonium sulphate (the resulting mixture is $2M$). Mix, filter through No. 44 Whatman filter-paper, and when the filtrate comes through clear refilter through the same paper. Measure 5 ml. of the filtrate into a centrifuge-tube and add 5 ml. 10 % trichloro-acetic acid. Continue as for total protein.

3. *Preparation of the standard.* Measure 5 ml. of the standard 0·24 % solution of protein into a centrifuge-tube, add 5 ml. 10 % $CCl_3.COOH$ and continue as for total protein.

Calculation. If the unknown is set at 20 mm. and the readings of the standard for total protein and albumin are X and Y mm. respectively, then

$$\begin{aligned} \text{Total protein} &= 0{\cdot}3X\ \%. \\ \text{Albumin} &= 0{\cdot}24Y\ \%. \\ \text{Globulin} &= (0{\cdot}3X - 0{\cdot}24Y)\ \%. \end{aligned}$$

Application of method to urine.

The following modifications are involved when the method is applied to urine:

1. The urine must be made faintly alkaline to litmus (about p_H 7·4).

2. The amount of urine for the determinations must vary with the total protein content as approximately determined (*e.g.* by Aufrecht's method).

When the protein content is in the region of 1 % (a figure not often exceeded in pathological urines), 1 ml. should be used for total protein, 3 ml. for albumin. When the protein content is low (about 0·25 %), the quantities used are 4 ml. and 12 ml. respectively.

At intermediate values corresponding quantities of urine are used.

3. Saturated ammonium sulphate (52·8 %) should be used for the albumin estimation, the volume added being equal to that of the urine. Half the filtrate may then be used as in the case of the serum method.

[1] Autenrieth states that shaking for 3 minutes is necessary for the maximum development of colour, but I have found no significant difference between shaking 5, 10, 30 and 180 seconds.

SUMMARY.

1. A modification is described of the biuret method of estimating serum proteins.

2. It is confirmed that the violet colour developed by equal amounts of albumin and globulin is practically the same, and that therefore serum may be used as the standard solution for estimating both total protein and albumin.

3. The standard advocated is serum diluted to contain 0·24 % protein. It keeps for several months at least in the presence of chloroform.

4. Ammonium sulphate (2 *M* finally) is used for the separation of globulin. The results of the albumin estimation do not correspond with those obtained when sodium sulphate (1·5 *M*) is employed and are usually higher. This is important as it has been claimed that fractionation of the protein is the same with both.

5. The modifications required for urine are described.

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